UNIVERSITY OF CINCINNATI

August 11 , 2003

I, Holly L. Allen ,

hereby submit this as part of the requirements for the degree of:

Master of Science (M.S.)

in Department of Molecular Genetics, Biochemistry, and Micro

It is entitled Development and Characterization of a Murine Model of Reactivation Histoplasmosis

Approved by:

DEVELOPMENT AND CHARACTERIZATION OF A MURINE MODEL OF REACTIVATION HISTOPLASMOSIS

A thesis submitted to the

Division of Research and Advanced Studies
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE (M.S.)

in the Department of Molecular Genetics, Biochemistry, and Microbiology
of the College of Medicine

2003

by

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Abstract

Histoplasma capsulatum (Hc) is a dimorphic fungus endemic to the Ohio River Valley area. In the immunocompromised patient, Hc infections can result from reactivation of a previous infection. In this project, we endeavored to develop a murine model of reactivation histoplasmosis and to characterize the subsequent host response to the disease. The results show that elimination of both CD4+ and CD8+ T cells in wildtype C57BL/6 mice induces reactivation of Hc organisms. Concomitant neutralization of Th1 cytokines alters the time course of disease, but does not significantly increase fungal burden. We also report that B cell deficient mice are significantly more susceptible to reactivation. Depletion of CD4+ T cells alone or in combination with CD8+ T cells is able to induce reactivation, while depletion of CD8+ has no effect. Thus, T cells appear to be the key players in the prevention of reactivation disease, while Th1 cytokines and B cells play a minor role.

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Abbreviations and Symbols

Abbreviations

AIDS – acquired immune deficiency syndrome

CFU – colony forming units

CMI – cell mediated immunity

GM-CSF – granulocyte/monocyte-colony stimulating factor

Hc – *Histoplasma capsulatum*

HIV – human immunodeficiency virus

IFN – interferon

IL – interleukin

mAb – monoclonal antibody

NK – natural killer

PMN – polymorphonuclear neutrophils

TCR – T cell receptor

TNF – tumor necrosis factor

Symbols

α – alpha

β – beta

γ – gamma

μ – mu

Introduction

Organism and Disease

Histoplasma capsulatum (Hc) is a dimorphic fungus that is endemic to the Ohio River and Mississippi River Valleys (2). It exists in the nonpathogenic mycelial phase in the natural environment (or 25°C *in vitro*) and in the pathogenic yeast phase inside the host (or 37°C *in vivo*) (3, 4). The mold phase of Hc consists of mycelia, macro-, and micro-conidia and is associated with rich soil, usually contaminated with bird or bat guano (5). Upon disruption of the natural environment, the conidia are aerosolized and inhaled into the bronchioles and alveoli of a mammalian host. It transforms into the pathogenic yeast phase approximately 10 hours later (3, 4, 6, 7). Once the organism is in the host, it is phagocytosed by resident macrophages, where it is able to survive intracellularly and replicate within the phagosome (8-10).

The most common route of infection with Hc is via the respiratory tract. While 99% of all individuals exposed to Hc develop acute pulmonary disease (6), the vast majority of cases seen in immunocompetent patients are resolved spontaneously and usually exhibit no symptoms (4, 6). If symptoms are present, they are frequently mild and influenza-like. Although it is rare, infection can also progress to chronic or progressive disseminated disease, where the organism is found not only in the lungs but also in multiple organs, including the spleen, liver, and lymph nodes (6). Infection with Hc is particularly problematic in immunocompromised patients, such as those with acquired immune deficiency syndrome (AIDS) or those undergoing immunosuppressive therapy.

Infection in these patients frequently causes acute disseminated disease, a life-threatening illness (11, 12).

There are two potential mechanisms of disease development in immunocompromised patients: dissemination of a newly acquired infection (from either a primary or secondary exposure) or reactivation of latent foci of infection (12). It is likely that the majority of cases of histoplasmosis in humans are the result of a newly acquired infection, particularly since exposure levels can be as high as 85% in endemic areas (13). However, there is mounting evidence that suggests that latent foci can reactivate in immunocompromised patients and cause disease. Development of histoplasmosis has been observed in patients who normally reside outside the endemic area but had traveled to endemic areas previously (14, 15). In addition, Hc organisms with mitochondial DNA patterns similar to those from Central American strains (and distinct from the two typical North American strains) have been isolated from HIV+ patients who had immigrated to the United States years before developing infection (16).

Immune Response to Hc

Cell-mediated immunity (CMI) is a critical host-defense mechanism that it activated in response to many intracellular pathogens. Once an organism is engulfed by an antigen-presenting cell (APC), antigens from the organism are processed and presented on the surface of the cell. Those antigens are recognized by T cells expressing the T cell receptor (TCR) α/β. The T cell:APC interaction leads to activation of the infected cell and resolution of disease. CMI is essential for the clearance of Hc organisms from the

mammalian host and has been well characterized in the murine model of histoplasmosis. Following infection with Hc yeasts, there is an influx of immune cells into the lungs of infected mice, including T cells, B cells, monocytes/macrophages, polymorphonuclear neutrophils, and natural killer cells (17). Along with the appearance of these immune cells, a number of cytokines also are produced. Th1 cytokines, which promote inflammation, macrophage activation, and killing of intracellular pathogens, have been found to be increased during acute infection while Th2 cytokines, which promote humoral immunity, are diminished (17, 18). The immune cell and cytokine influx during primary Hc infection is critical to resolution of disease and perturbation of any of these factors can negatively affect host defense.

The presence of T cells is a critical part of host resistance to Hc. Mice treated with mAb to T cell receptor (TCR) α/β are more suseptible to primary Hc infection compared to untreated mice and eventually die by 12 days post-infection (18). α/β TCR+ T cells can be divided into two basic subsets—CD4+ and CD8+, both of which contribute to host defense. Mice depleted of CD4+ T cells exhibit increased fungal burden and ultimately succumb to infection by 20 days post-infection (18, 19). Mice depleted of CD8+ T cells survive infection, but are more susceptible to disease and take longer to clear infection compared to CD8-sufficient animals (18, 20). Additionally, transfer of CD4+ and not CD8+ T cells from Hc-immunized mice can confer protection on naive animals (21). In Hc-immune mice, the presence of either CD4+ or CD8+ T cells is sufficient to survive infection and only depletion of both subsets causes mortality (18). These data indicate that CD4+ T cells are essential and CD8+ T cells are optimal for clearance of the

organism in primary infection, while either subset is sufficient for clearance in secondary infection.

The immune reponse to Hc infection has been well characterized in C57BL/6 mice. The major mechanism by which T cells mediate protective immunity to Hc infection is via production of proinflammatory cytokines and the subsequent activation of macrophages, which serves to restrict intracellular growth of the organism. Three important cytokines that participate in host defense are IFNγ, TNFα, and GM-CSF.

Interferon gamma (IFNγ)

IFNγ has been shown to activate murine peritoneal macrophages and upregulate inducible nitric oxide synthase, resulting in the production of nitric oxide and inhibition of intracellular growth of Hc (22, 23). Elimination of IFNγ either by treatment with a neutralizing monoclonal antibody (mAb) or disruption of the IFNγ-gene in mice results in increased mortality and higher Hc fungal burden compared to control mice in both primary and secondary infection (24, 25).

Tumor necrosis factor alpha (TNFα)

TNFα has been shown to have varying effects on both primary and secondary infections. Neutralization of TNFα in both primary and secondary Hc-infected mice results in increased fungal burden and 100% mortality (26, 27). In primary infection, this effect correlates with a decrease in the production of reactive nitrogen intermediates although treated mice do still produce adequate amounts of IFNγ and other Th1 cytokines (27).

This is not the case in secondary infection. Here, the increase in fungal burden is associated with a predominant Th2-response, which can be reversed by neutralizing IL-4 and IL-10 (27).

Granulocyte/monocyte – colony stimulating factor (GM-CSF)

CSFGM-CSF is also involved in the host response to Hc infection in mice. Neutralization of GM-CSF during primary Hc infection results in an 80% drop in survival and a failure to control growth of the organism. This was associated with a decrease in IFNγ and TNFα production as well as a decrease in reactive nitrogen intermediates (28). Futhermore, adminstration of recombinant GM-CSF to Hc-infected mice has been shown to improve survival of T cell-depleted mice exposed to Hc (29), indicating the importance of GM-CSF in early immunity to Hc infection.

An important aspect of host immunity against pulmonary intracellular pathogens, including Hc, is the development and maintainance of granulomas (reviewed in (30) and shown in Figure 1.) The granuloma is a cohesive, organized structure designed to contain

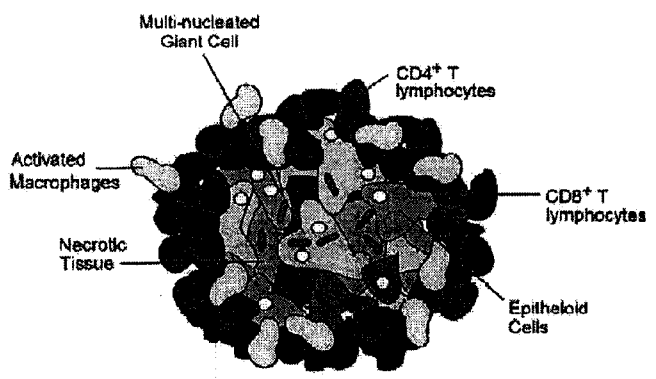

Figure 1: A typical pulmonary granuloma (1).

T cells surround infected phagocytes and form a structure designed to prevent dissemination of the organism.

and prevent the dissemination of the pathogen. During the immune response to a intracellular pathogen, T cells are recruited to the site of infection. Upon continuous exposure, there is excessive production of fibronectin and collagen. These materials weave together with immune cells and epithelial tissue to form a tight, well-defined structure in which T cells surround infected macrophages. These infected macrophages can fuse together to form multi-nucleated giant cells or can become necrotic. In either case, organisms are effectively "walled-off" from healthy tissue and dissemination is prevented. However, organisms can remain viable within the granuloma and organisms from several pathogenic infections have been cultured from loci that were previously thought to be fully healed (12).

Goals and Experimental Design

Previous research on the immune response to Hc has primarily focused on the use of murine models of primary and secondary Hc infection. Use of these models has indicated that the immune cell and cytokine influx during Hc infection is critical to resolution of disease. However, in the immunocompromised patient, it is possible that reactivation of latent foci of infection can cause disease, which is neither a primary nor a secondary infection. Therefore, primary and secondary murine models of disease cannot be used to study this mechanism of host defense. Little to no information on the mechanism by which the host prevents reactivation histoplasmosis is available, and no concrete mouse model of reactivation has been developed. Durkin et. al have shown persistant histoplasmosis in immunocompetent B6C3F1 mice infected with a clinical strain of Hc, 50% of which spontaneously reactivate between weeks 26 and 56 post

infection (31). However, the immune response to Hc in those mice has not been well characterized and the mechanisms by which reactivation histoplasmosis occus are unknown. To that end, we have endeavored to develop a murine model of disease in order to characterize the host response to reactivation histoplasmosis.

We have developed a model of reactivation histoplasmosis based on information already gathered about the immune response to Hc during primary and secondary infection. We have previously shown that CD4+ T cells are essential, while CD8+ T cells are optimal for clearance of the organism in primary infection, while either subset is sufficient for clearance in secondary infection (32). Since both subsets are involved in clearance of both primary and secondary infection, it is likely that they are also involved the host reponse to reactivation histoplasmosis. We also wanted to know whether neutralization of pertinent Th1 cytokines would enhance reactivation of Hc organsisms, since they are also involved in both the primary and the secondary host response (24-27). To answer these questions, we used neutralizing mAb to remove specific T cell subsets and cytokines from the host in order to determine any effect they may have on immunity. This is a method that has been successfully used in our laboratory in the past (27, 28, 32). In order to mimic reactivation histoplasmosis in humans, we began our mAb treatments after the mice had resolved the orginal primary infection. No organisms could be detected from the lungs or spleens (at least six weeks following infection.) In modifying the primary and secondary models of Hc infection, we have developed a very powerful tool by which many questions about the immune response to Hc infection can be answered.

Results

A. Role of CD4+ and CD8+ T cells and Th1 cytokines in reactivation histoplasmosis

T cells and Th1 cytokines have been shown to be very important in the host response to infection with Hc. Therefore, we hypothesized that they would also be involved in the control of reactivation histoplasmosis. To test this, groups of mice were infected with 2x106 Hc yeasts. Six weeks post infection, when there were no detectable yeasts in the lungs or spleens (data not shown), mice were treated with 100 μg of mAb to both CD4 and CD8 alone or in combination with 1 mg each of anti-TNFα and anti-IFNγ for two weeks. At this time, mice were sacrificed and the fungal burden in the lungs and spleens was determined. Depletion of CD4+ and CD8+ T cells alone or in combination with neutralization of TNFα and IFNγ induced reactivation of Hc in both organs, while mice treated with an irrelevant rat IgG exhibited no detectable CFU (Figure 2.) TNFα and IFNγ neutralization did have an effect when combined with CD4+ and CD8+ T cell depletion in the spleens, as shown in Figure 2B. The mean $\log_{10}$ CFU ± SEM was significant increased in the spleens of mice treated with all four antibodies (3.78 ± 0.209) compared to mice depleted of T cells alone (2.60 ± 0.230.) This is not the case in the lungs of the same animals. Although there was no significant difference between the two groups in the lungs, as shown in Figure 2A, fungal burden appeared to be lower in mice treated with all four antibodies (2.60 ± 0.189) compared to mice depleted of T cells alone (3.32 ± 0.492).

To determine whether reactivation was due to effects by a single T cell population or a single cytokine, infected mice were treated individually with mAb to CD4, CD8, TNFα,

or IFNγ. At 8 weeks post infection, only mice that were depleted of both CD4+ and CD8+ T cells exhibited significant ($p<0.001$) reactivation of Hc yeasts in the lungs and spleens (Figure 3A and 3B.) Neutralization of IFNγ did induce a low level of reactivation (2.08 ± 0.225) of Hc organisms in the lungs of one animal. This value is very close to the limits of detection for this assay and is not significantly different from rat Ig-treated animals. Thus, depletion of CD4+ and CD8+ T cells with or without the added neutralization of TNFα and IFNγ was associated with the reactivation of Hc yeast in previously challenged mice.

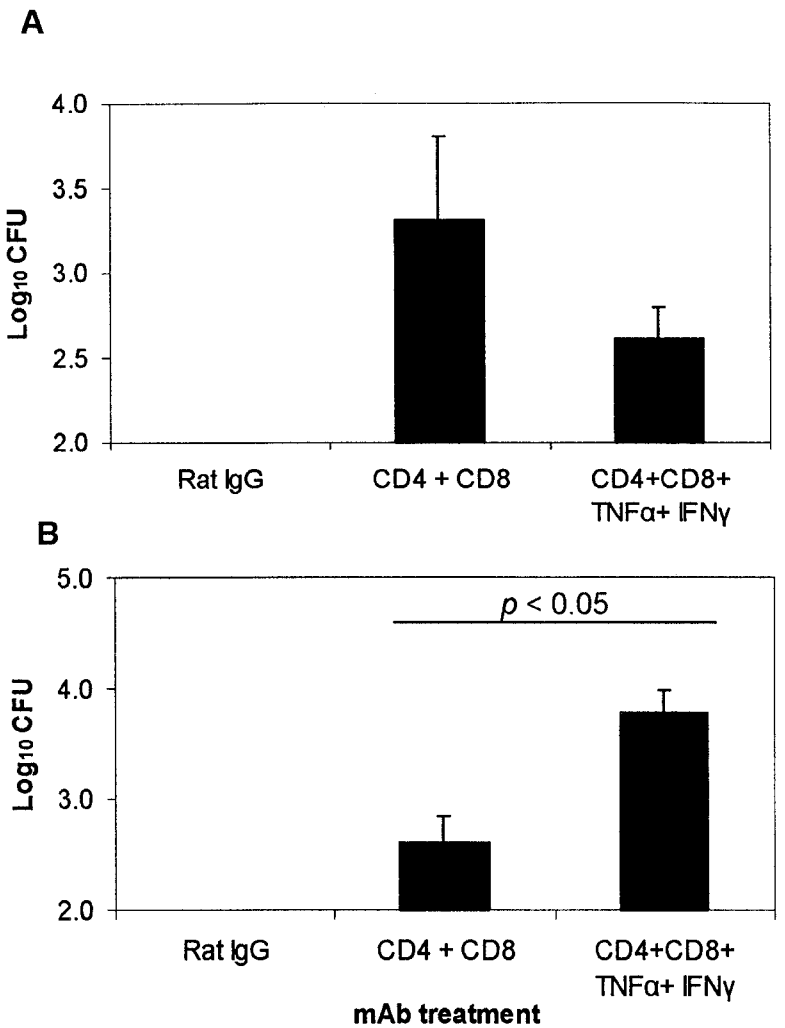

Figure 2: Concomitant depletion of CD4+ and CD8+ T cells and neutralization of TNFα and IFNγ. C57BL/6 mice (n=5-6/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4 and CD8 alone, with mAb to CD4, CD8, TNFα, and IFNγ or with an equal amount of rat IgG. Two weeks following initial depletion, the mice were sacrificed and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. Both groups are significantly different ($p<0.001$) from rat IgG controls.

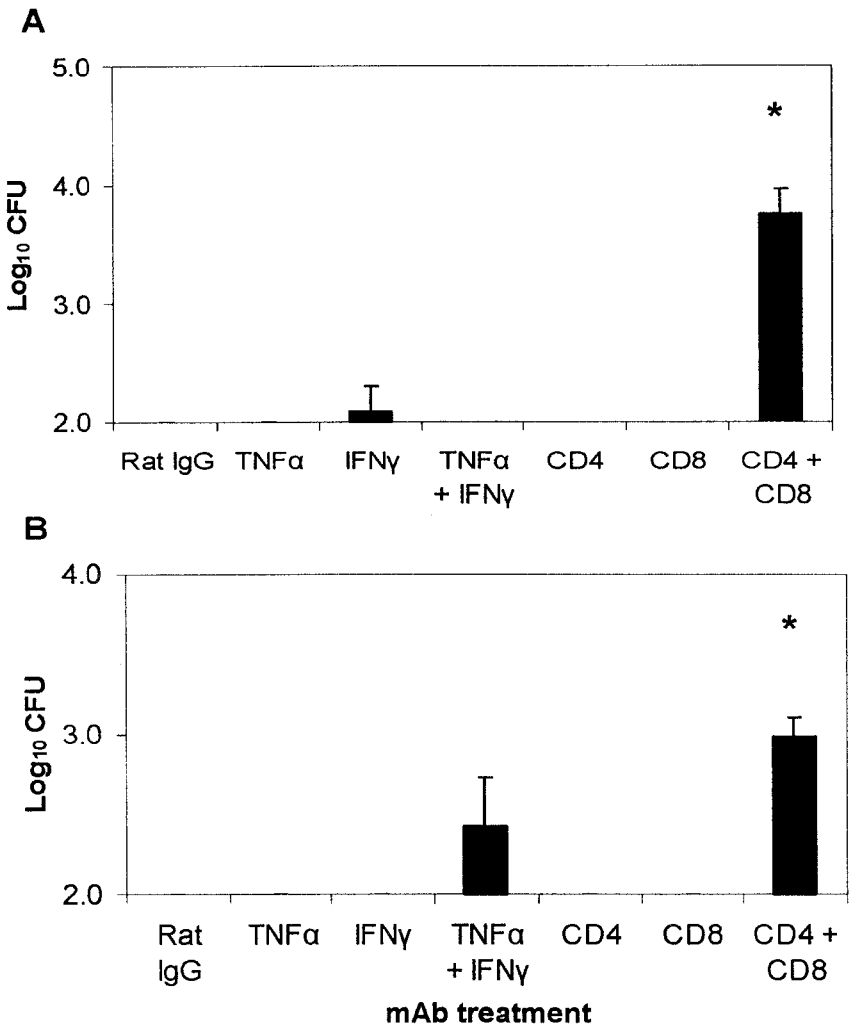

Figure 3: Individual depletion of CD4+ or CD8+ T cells and neutralization of TNFα, or IFNγ. C57BL/6 mice (n=4/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4, CD8, TNFα, or IFNγ alone. Two other groups of mice were treated with mAb to CD4 and CD8 or with mAb to TNFα and IFNγ. A control group of mice was treated with rat IgG. Two weeks following initial depletion, the mice were sacrificed and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. * = $p<0.001$

B. Time course of reactivation histoplasmsosis

To determine whether our model of disease was chronic or acute, we examined the effects of CD4+ and CD8+ T cell depletion and TNFα and IFNγ neutralization over time. Mice were treated weekly with mAb beginning six weeks post infection, sacrificed at two-week intervals and fungal burden was assessed in the lungs and spleens. Reactivation of Hc yeasts was evident in mice treated with mAb to CD4 and CD4 alone or in combination with mAb to TNFα and IFNγ (Figure 4). Fungal burden in these groups of mice remained higher than mice treated with rat IgG at all time points. However, there were significant differences ($p<0.05$) between the two treated groups of mice at several time points. Fungal burden in mice treated only with anti-CD4 and anti-CD8 peaked at 8 weeks in the lungs and became steady thoughout the rest of the experiment. Fungal burden remained constant in the spleens at all time point, although Hc yeasts were always detectable. Mice treated with all four mAbs peaked at 10 weeks post infection. Most importantly, there are no significant differences in fungal burden in between both groups of mice in either lungs or spleens in the later stages of the time course. Inclusion of mAb to GM-CSF to the neutralization treatments did not significantly change this trend, with the exception of the week 12 time point (Figure 5.) Thus, depletion of CD4+ and CD8+ T cells induces chronic reactivation of histoplasmosis. Neutralization of Th1 cytokines in addition to T cell depletion appears to only shift the time course of reactivation of Hc yeasts rather than cause a dramatic change in fungal burden. It is interesting to note that depletion of CD4+ and CD8+ T cells has a different effect in the lungs versus the spleens of infected mice. In the lungs (Figure 4A and 5A), fungal burden peaks early in the time course of infection and then decreases to

barely detectable. In the spleens (Figure 4B and 5B,) fungal burden remains constant throughout the time course. In either organ, additional treatment with mAb to TNFα, IFNγ and/or GM-CSF only shifts these trends.

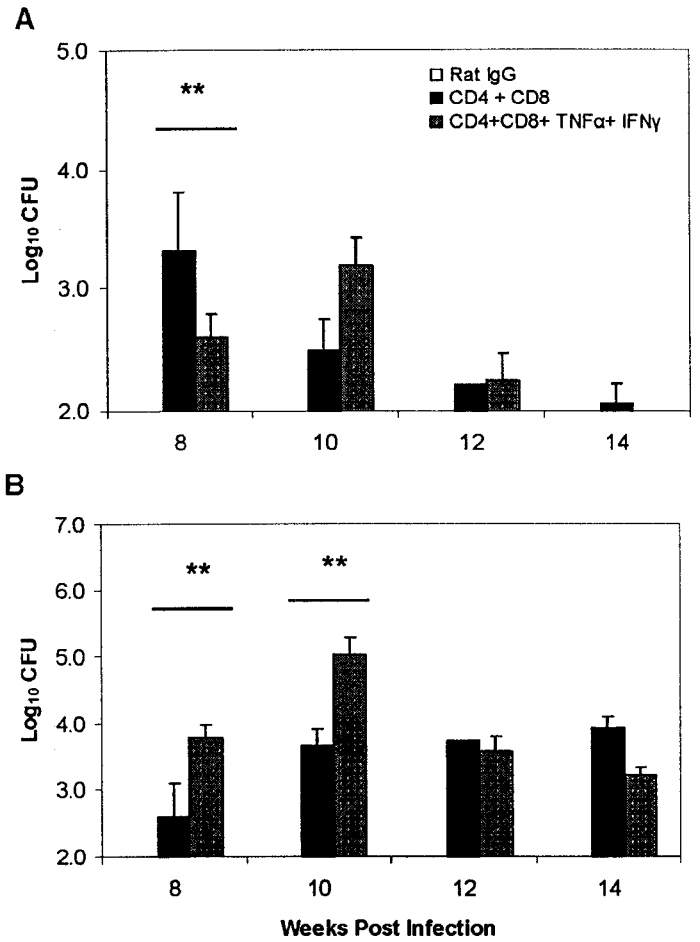

Figure 4: Time course of reactivation histoplasmosis induced by simultaneous neutralization of TNFα and IFNγ and depletion of CD4+ and CD8+ T cells.

C57BL/6 mice (n=6-10/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4 and CD8 alone or with mAb to CD4, CD8, TNFα, and IFNγ. A control group of mice was treated with rat IgG. The mice were sacrificed at two week intervals and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. Both groups are significantly different ($p<0.001$) from rat IgG controls, but not from each other with the exception of the following: * = not significant from control, ** $p<0.05$

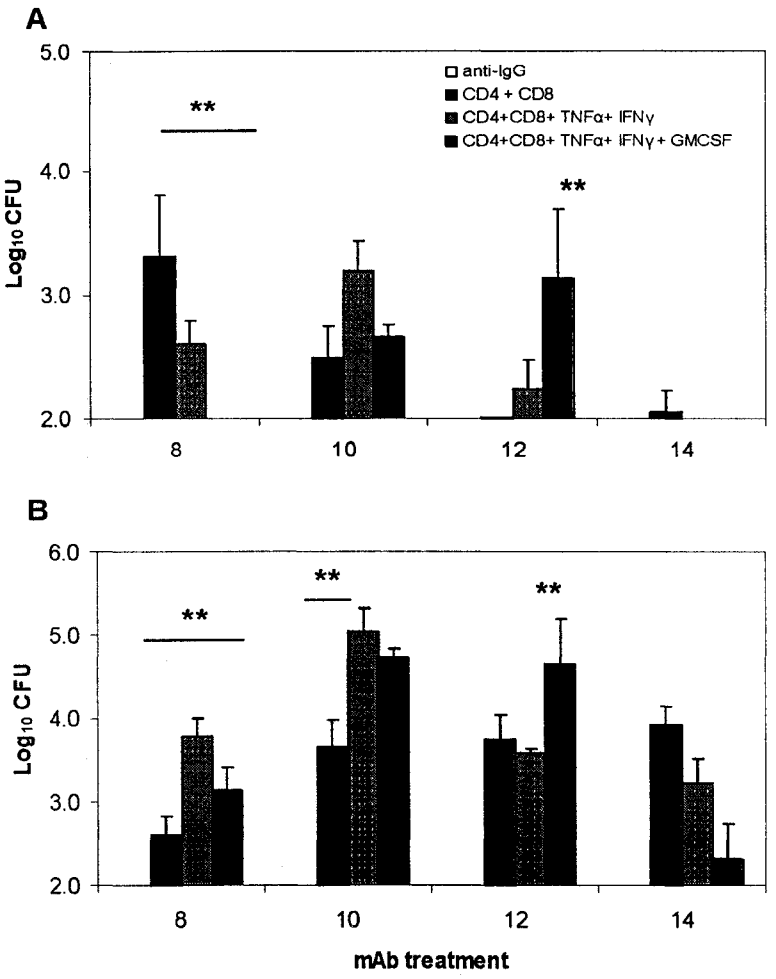

Figure 5: Simultaneous neutralization of Th1 cytokines and depletion of CD4+ and CD8+ T cells. C57BL/6 mice (n=6-10/group) were infected i.n. with $2x10^6$ Hc yeasts Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4 and CD8 alone or with mAb to CD4, CD8, TNFα, IFNγ, and GMCSF. A control group of mice was treated with rat IgG. The mice were sacrificed at two week intervals and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. All groups are significantly different ($p<0.001$) from rat IgG controls, but not from each other with the exception of the following: * = not significant from control, ** $p<0.05$

C. Reactivation histoplasmosis in B cell deficient mice

In this study, we treated mice with neutralizing rat mAb continuously over a 6 week period in order to remove T cells and cytokines from the host during infection. A potential problem with this method is that the treated mice may produce antibodies against the rat mAbs, which may affect their depleting capabilities. One way to test this possibility is to use B cell deficient mice (μMT) that do not make B cells or antibody, thus eliminating the possibility of neutralization and subsequent decreased function of our treating antibodies. The B cell–deficient μMT mouse carries a stop codon and the neomycin gene cassette in the 5' end of the first transmembrane exon of the μ chain, which causes a developmental block at the pre- B cell stage (33). These mice lack cells that express IgM or IgD, resulting in a severe reduction in mature, antibody-producing B cells. In these experiments, μMT mice and their wildtype controls were treated weekly with mAb to both CD4 and CD8 beginning six weeks post infection and were sacrificed at two week intervals. Fungal burden was assessed in the lungs and spleens. Surprisingly, there was a significant increase ($p<0.001$) in mean $\log_{10}$ CFU ± SEM in B cell deficient mice beginning at 10 weeks and 12 weeks (3.3 ± in the lungs 6.25 ± 0.19 in the spleens) post infection compared to wildtype control mice at the same time points (2.10 ± 0.10 in the lungs and 3.4 ± 0.39 in the spleens) (Figure 6.) B cell deficient and wildtype mice treated with irrelevant rat IgG mAb did not exhibit detectable CFU at any time point (data not shown.)

To determine if this effect was due to decreased efficacy of our depleting rat mAbs or due to B cells themselves, splenocytes from mice sacrificed 14 weeks post infection were

stained with FITC-conjugated antibodies to B220/CD45 (a common B cell marker,) CD4, or CD8 and analyzed by flow cytometry (Figure 6.) In figure 6A, μMT mice exhibited a decrease in B cell production to below 10% in both control and CD4/8 depleted groups, indicating that both groups of mice are indeed deficient in B cell production. In figure 6B, CD4/8 depleted μMT and wildtype mice show a sharp decrease in CD4+ and CD8+ T cells compared to rat IgG treated controls. This observation at the 14 week time point indicates that rat mAb is efficacious for depleting T cells in a long-term depletion experiment. Further, the differences in fungal burden observed in B cell deficient mice indicate that B cells and/or antibody play a role in reactivation histoplasmosis.

To further tease apart the roles of B cells and T cells in reactivation histoplasmosis, Hc-infected μMT mice were treated individually with mAb to CD4, CD8, or CD4 and CD8 combined, beginning at 6 weeks post infection. As we had previously observed at 12 weeks post infection, mice depleted of both CD4+ and CD8+ T cells showed a significant increase in fungal burden ($p<0.001$) compared to control mice (Figure 8.) Interestingly, mice depleted of only CD4+ T cells also exhibited a significant increase ($p<0.001$)in the mean log10 CFU (3.20 ± 0.09 in the lungs and 3.86 ± 0.20 in the spleens), although not as high as that seen in the double depleted mice (4.03 ± 0.22 in the lungs and 5.19 ± 0.55 in the spleens). This result was unexpected since only depletion of both CD4+ and CD8+ T cells was able to induce reactivation of Hc yeasts in wildtype mice. We were unable to detect any Hc yeasts in organs from mice treated with mAb to CD8 at any time during the experiment.

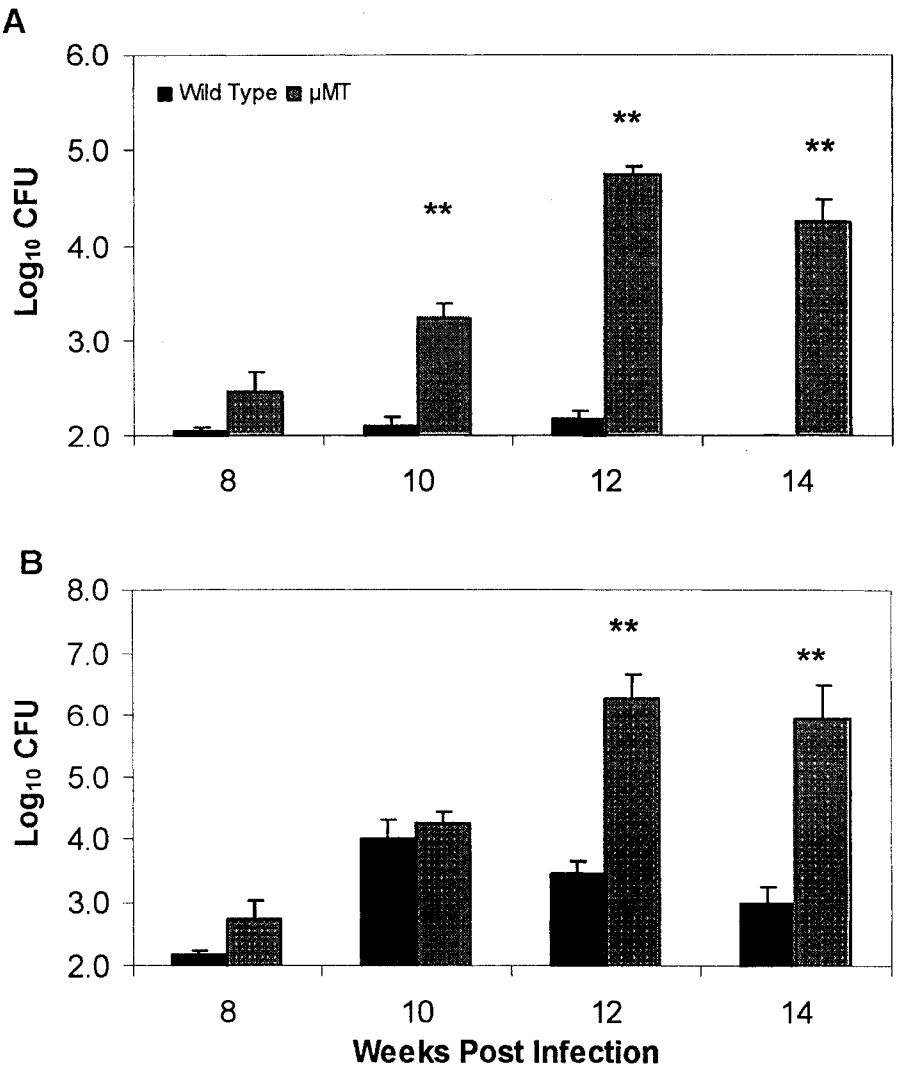

Figure 6: Concomitant depletion of CD4+ and CD8+ T cells in B cell knockout mice. C57BL/6 and µMT mice (n=6/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4 and CD8. Control group of both types of mice were treated with rat IgG. The mice were sacrificed at two week intervals and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. ** $p<0.001$

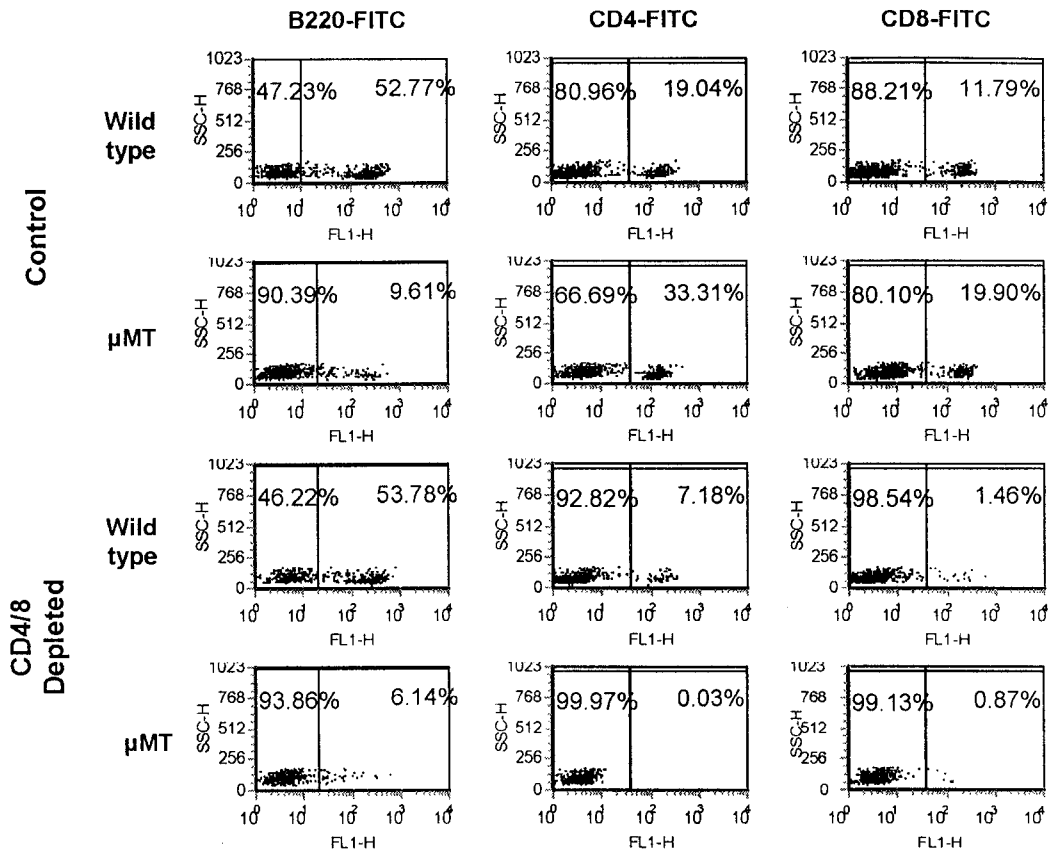

Figure 7: Phenotype of splenocytes isolated from wildtype and B cell knockout mice. C57BL/6 and µMT mice (n=6/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4 and CD8. Control group of both types of mice were treated with rat IgG. The mice were sacrificed at 14 weeks post infection and splenocytes were isolated. Cells were stained with B220-FITC, CD4-FITC, or CD8-FITC. Shown cell populations were gated on live, intact cells.

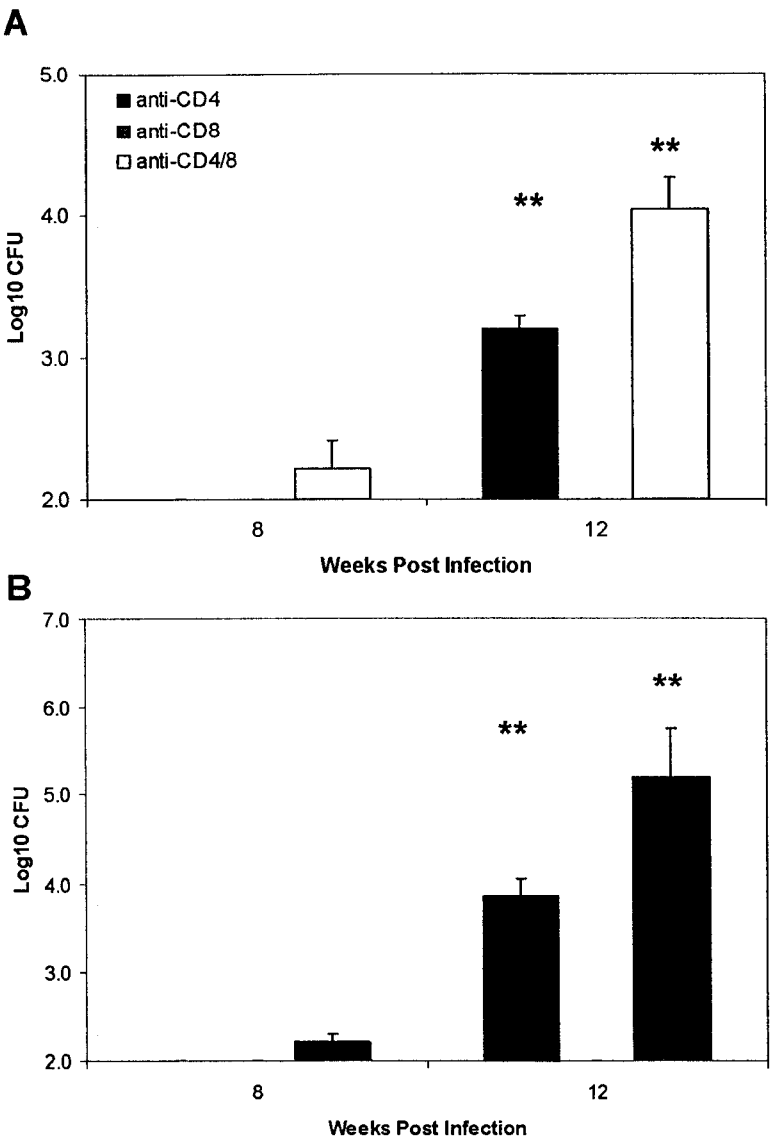

Figure 8: Individual depletion of CD4+ and CD8+ T cells in B cell knockout mice. C57BL/6 and μMT mice (n=6/group) were infected i.n. with $2x10^6$ Hc yeasts. Beginning six weeks post-infection, mice were treated weekly with mAb to CD4, CD8, or CD4 + CD8. Control group of both types of mice were treated with rat IgG. The mice were sacrificed at two week intervals and fungal burden was evaluated in the lungs (A) and spleens (B). Data are expressed as the mean CFU ± SEM. ** $p<0.001$

Discussion

In primary and secondary Hc infection, T cells and Th1 proinflammatory cytokines are essential for clearance of the organism and resolution of disease. Little is known about the requirements for the prevention of reactivation histoplasmosis. The purpose of this study was to develop a murine model of reactivation disease and to characterize the subsequent host response. The results show that elimination of both CD4+ and CD8+ T cells in wildtype C57BL/6 mice induces reactivation of Hc organisms over a six week time period. Fungal burden peaks early during the reactivation process in both the lungs and spleens and then decreases to a steady level and organisms are detectable throughout the entire depletion process. Concomitant neutralization of important Th1 cytokines, TNFα, IFNγ, and GM-CSF does not significantly increase reactivation, although it does alter the time course of reactivation. We have also shown that C57BL/6 B cell deficient mice are significantly more susceptible to reactivation histoplasmosis compared to their wildtype counterparts. In these mice, depletion of CD4+ T cells alone or in combination with CD8+ T cells is able to induce reactivation of Hc organisms, while depletion of CD8+ has no effect. Thus, T cells appear to be the key players in the prevention of reactivation, while Th1 cytokines and B cells/antibody play a minor role.

Although not studied extensively in Hc infection, reactivation or latent disease caused by other intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania major* has been well documented. Depletion of either CD4+ (34) or CD8+ (35) T cells have been shown to cause reactivation of disease, primarily via diminished production of TNFα and IFNγ. The decreased cytokine production leads to abrogation of important

downstream effects such as granuloma formation, leukocyte recruitment, and macrophage activation and dissemination of bacteria (34-38). This observation has been supported by reports showing that neutralization of TNFα (37, 39, 40) or IFNγ (35, 40), or inhibition of nitric oxide production (41-43) have all been shown to reactivate disease. Additionally, *in vivo* production of IL-10 during latent tuberculosis has also been shown to reactivate *M. tuberculosis*, even in the presence of CD4+ and CD8+ T cells (44). In that study, increased production of IL-10 was associated with a decrease in TNFα, IFNγ, and IL-12p40. The importance of IL-10 in the prevention of persistant infection was also shown by Belkaid et al. in which mice treated with anti-IL-10 receptor antibodies were able to achieve sterile cure of leishmaniasis while untreated mice suffered from persistent disease (44, 45). These data suggest that the critical players in reactivation of other intracellular pathogens are Th1 cytokines, rather than T cells. This does not appear to be the case in our model of reactivation histoplasmosis, in which depletion of both CD4+ and CD8+ T cells are necessary before any Hc any organisms could be detected and neutralization of Th1 cytokines has only minimal effects.

The mechanism by which CD4+ and CD8+ T cells mediate control against reactivation of Hc organisms in the absence of key proinflammatory Th1 cytokines remains unknown. There are several possible explanations for this observation. One possible explanation is the regulation of granuloma formation and maintenance. Granulomas are formed during the immune response to most intracellular pathogens, including Hc infection (30). T cells are critical in the formation of granulomas not only because they produce Th1 cytokines but also because they form a physical barrier between organism and healthy tissue in the

infected host. It is possible the act of removing this barrier via depletion of T cells is sufficient to allow dissemination and growth of the organism. Since T cells are major producers of Th1 cytokines during the infectious processes, depletion of them may also aborgate the majority of the cytokine production during reactivation, making neutralization of Th1 cytokines appear ineffective. This would support our observation that neutralization of TNFα, IFNγ, and GMCSF in combination with CD4+ and CD8+ depletions has only minimal effects. Another explanation for our results is the possibility that CD4+ and CD8+ T cells have both overlapping and distinct functions, both of which are important for successful control. For example, both CD4+ and CD8+ T cells are able to produce IFNγ during *M. tuberculosis* latent infection. Although depletion of CD4+ T cells leads to reactivation of bacteria, it does not lead to a reduction in IFNγ production, while depletion of CD8+ T cells do (35), suggesting that both T cells subsets have functions that are important to the prevention of reactivation disease. Our data suggests that during Hc reactivation disease, CD4+ and CD8+ have overlapping functions, such that only depletion of both subsets causes disease.

One caveat to using mAb during a long-term *in vivo* experiment is the possibility that the treated mice may produce antibodies against the rat mAbs, thus affecting their depleting capabilities. One way to test this possibility is to use B cell deficient mice that do not make B cells or antibody. The B cell–deficient μMT mouse carries a stop codon and the neomycin gene cassette in the 5' end of the first transmembrane exon of the μchain, which causes a developmental block at the pre- B cell stage (33). These mice lack cells that express IgM or IgD, resulting in a severe reduction in mature, antibody-producing B

cells. Our laboratory has previously shown that B cell deficient (μMT) mice have equivalent fungal burdens and clear Hc infection just as efficiently as wildtype mice in both primary and secondary infection (18). To that end, we hypothesized that B cell deficient mice would behave similarly to wildtype mice and that our neutralizing mAb antibodies would be efficacious throughout the entire experiment. FACS analysis of splenocyte phenotype showed that μMT mice had a severe reduction in B220+ B cells and mice treated with mAb against CD4 and CD8 remain depleted of those cells types throughout the 14 weeks experiment. Surprisingly, B cell deficient mice are significantly more susceptible to reactivation histoplasmosis compared to wildtype mice. Not only do they have increased fungal burden compared to wildtype mice when depleted of both CD4+ and CD8+ T cells, but they also reactivate when depleted of only CD4+ T cells. Depletion of CD8+ T cells has no effect on fungal burden. This is a different observation than seen in wildtype mice, where both CD4+ and CD8+ T cells must be depleted in order of Hc organisms to reactivate. These data suggest that B cells and/or antibody do indeed play role in Hc infection, at least in the control of reactivation.

It was once thought that B cells and antibody played little to no role in the host defense against intracellular organisms since antibody is unable to reach the organism within the host cells. However, a growing body of evidence suggests that B cells themselves have pleiotropic effects on the immune reponse and are able to make some contibution to immunity against intracellular pathogens, even in the absence of antibody. These effects include regulation of the proinflammatory response, T cell interaction/ activation, and antigen presentation. During *Cryptococcus neoformans* infection in mice, administration

of a mAb specific to the capsule of the organism is able to prolong survival and enhance the granulomatous response without altering fungal burden, via the downregulation of the proinflammatory response and prevention of the destructive pathology associated with Th1 cytokine production (46-48). The use of mice deficient in FcγR1 and III (the receptors by which antibody mediates its neutralization/depletion functions) has shown that the clearance of *Pneumocystis carinii* in mice is dependent on B cells and not antibody (49). In that study, B cell deficient mice had reduced numbers of activated CD4+ cells in the lungs and lymph nodes of infected mice. The presence of functional mAb during *Chlamydia trachomatis* infection is associated with enhanced chlamydial antigen presentation and specific antichamydial antibodies were shown to augment macrophage killing and inhibit chlamydial growth (50).

Our experiments show that depletion of CD4+ and CD8+ T cells together are required to reactivate Hc organisms in wild-type mice, while depletion of CD4+ T cells alone are sufficient in B cell deficient mice. These data suggest that B cells and/or antibody play a role in reactivation histoplasmosis. There are two potential mechanisms by which B cells can mediate protective immunity to Hc—antibody production or antibody-independent B cell functions such as cytokine production or T cell interactions. Antibody production is almost entirely dependant on CD4+ T cells. Johnson and Sayles showed that CD4 deficient mice are much more susceptible to a virulent strain of *Toxoplasma gondii*, in part because the levels of *Toxoplasma*-specific immunoglobulin G2a in serum were substantially lowered. Passive transfer of immune serum was able to restore immunity to the pathogen (52). However, it is unlikely that lowered antibody levels is an explanation

for our results, since elimination of CD4+ T cells alone in B cell sufficient mice had no effect on fungal burden. Another possible explanation for our results is the possibility that in the absence of B cells, CD8+ T cells are altered or eliminated. This hypothesis is supported by the report from Shen et al. showing that B cells play a role in the contraction phase of immune response to the intracellular pathogen *Listeria monocytogenes*. The absence of B cells results in increased death of CD8+ T cells following initial infection with *L. monocytogenes* and subsequent lower levels of Ag-specific CD8+ T cell memory (51). This phenomenon is not associated with any defect in CD4+ T cells function. It is possible that, similar to *L. monocytogenes* infection, CD8+ T cells numbers are also decreased during the contraction phase of the immune response to Hc in B cell deficient mice. This could potentially lead to ineffective granuloma formation, since both CD4+ and CD8+ T cells form the physical barrier around infected macrophages (30). Thus, depletion of CD4+ T cells alone would be sufficient to cause a significant reactivation of Hc organisms in B cell deficient mice while in wildtype B cell sufficient mice, the higher numbers of functional CD8+ T cells would be available to control infection.

Summary and Future Directions

The immune response to Hc infection following both primary and secondary exposure is dependent on the presence of T cells and proinflammatory Th1 cytokines. The mediators responsible for the control of reactivation histoplasmosis are currently unknown. We have developed a model of pulmonary reactivation histoplasmosis, in which CD4+ and CD8+ T cells are required for prevention of disease. The proinflammatory cytokines TNFα, IFNγ, and GM-CSF appear to play a miminal role. The exact mechanism by which T cells can mediate protective immunity to Hc in the absence of Th1 cytokines is unknown. Histological analysis of tissue samples from reactivated mice is currently underway. This information will lead to insight about the role of CD4+ and CD8+ T cells in granuloma formation and maintenance during reactivation histoplasmosis and will support our hypothesis that granuloma formation is critical to reactivation control.

We are also further investigating the role both proinflammatory and immuno-suppressive cytokines in reactivation disease. Quantification of cytokine messenger RNA transcripts in the organs of reactivated mice will further clarify the importance of cytokines in this process. Specifically, the role of IL-10 is also being investigated, since it has been shown that production of IL-10 can skew the Th1:Th2 balance and cause the reactivation of other intracellular organisms such as *Mycobacterium tuberculosis* and *Leishmania major* (44, 45). We have preliminary data showing that IL-10 production is increased during reactivation histoplasmosis (data not shown). The presence of IL-10 during reactivation may have an impact on the production of Th1 cytokines and may explain the lack of

effect of Th1 cytokine neutralization. We are currently using IL-10 knockout mice to further investigate the role of IL-10 in reactivation histoplasmosis.

We have also discovered a role for B cells and/or antibody in the immune response to Hc, since B cell deficient mice are significantly more susceptible to reactivation disease compared to wildtype mice. We will determine which of these mechanisms are at work in our model of reactivation using transfer experiments. In one experiment, mAb or immune serum is passively transferred into B cell deficient mice. In another experiment, purified B cells from immune and naïve animals are adoptively transferred into B cell deficient mice. These types of experiments will help clarify the role of antibodies versus antibody-independent B cell functions in Hc reactivation. Quantification and identification of immune cell type via FACS analysis, as well as histological analysis, will also be useful in elucidating the function of CD4+ and CD8+ T cells in B cell deficient versus wildtype mice.

Methods and Materials

Animals

Five-week old male C57BL/6 mice and μMT mice (strain B6.129S2-*Igh-6tm1Cgn*) were purchased from The Jackson Laboratory (Bar Harbor, ME). Five-week old male athymic nude mice were purchesed from the National Cancer Institute (Fredrick, MD) and used to produce ascites. Animals were housed under barrier conditions and maintained by the Department of Laboratory Animal Medicine at the University of Cincinnati (Cincinnati, OH), which is accredited by the American Association for the Accreditation of Laboratory Animal Medicine. All animal experiments were performed in accordance with Animal Welfare Act guidelines of the National Institutes of Health.

Hc culture and infection of mice

Yeast phase Hc (strain G217B) was used in all experiments. Yeasts were maintained by biweekly subculture on blood agar slants (Gibco BRL, Grand Island, NY) at 37°C. To prepare Hc yeasts for infection, cells were washed from the slants with Hanks' balanced salt solution (HBSS) (Biowhittaker, Walkersville, MD) and enumerated. Liquid cultures were seeded at 3 x 10^6 yeasts/ml into 50 ml of Ham's F12 media (Gibco BRL), pH 7.5, supplemented with glucose (18.2 g/L), glutamic acid (1 g/L), HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (6 g/L) and cysteine (8.4 g/L). Cells were incubated for 36 hours at 37°C in a rotating water bath (200 rpm). Cells were harvested from the media by centrifugation for 5 minutes at 800 xg, washed in HBSS, and enumerated. Mice were anesthetized with isofluorane and infected i.n. with a sublethal dose (2 x 106 yeasts) in a 30 μl volume of HBSS.

Organ Cultures

Mice were sacrificed at specified time points and the lungs and spleens were harvested under sterile conditions. Organs were homogenized in 5 ml of HBSS supplemented with gentamicin (10 mg/L) to prevent bacterial growth. Ten-fold dilutions of organ homogenates were plated on BBL™ Mycosel™ Agar (Becton Dickenson Company, Sparks, MD) supplemented with agarose (8 g/L), dextrose (10 g/L), cysteine (100 μg/L), gentamicin (5 mg/L), and defibrinated sheep blood (50 ml, Colorado Serum Company, Denver, CO). Plates were incubated at 30°C for 7 days or until fungal colonies could be enumerated. Colony counts are expressed as mean $\log_{10}$ colony forming units (CFU)/organ ± standard error (SE). The limit of detection for this assay is 10^2 CFU.

Preparation of mAb

The following monoclonal antibodies (mAb) were used in depletion and neutralization experiments: anti-CD4 (clone GK1.5, rat IgG2b), anti-CD8 (clone 2.43, rat IgG2b), anti-TNFα (clone XT22.11, rat IgG1), anti-IFNγ (clone XMG 2.1, rat IgG1), anti-GM-CSF (clone MPI-22E9, rat IgG2a), and anti-IL-10 (JES-2A5, rat IgG2b). XT22.11, XMG 2.1, and JES-2A5 were kindly provided by Dr. J. Abrams (DNAX Research Institute, Palo Alto, CA). Hybridomas were maintained in RPMI-1640 with 25 mM HEPES and L-glutamine, supplemented with Non-essential amino acids (NEAA) Mix (10 ml/L), Pen/Strep/Fungizone Mix (10 ml/L), L-glutamine (2 mM), sodium pyruvate (1 mM), gentimicin (10 mg/L), 2-mercaptoethanol (4 μl/L), and heat-inactivated fetal bovine serum (5% final volume, Atlanta Biologicals, Norcross, GA). Media and supplements

were purchased from Biowhittaker unless otherwise specified. mAb was produced by injecting 2.5 x 10^6 hybridoma cells i.p. into athymic nude mice and harvesting ascites or by by harvesting tissue culture supernatants. mAb was purified from ascites and tissue culture supernatants using a protein-G sepharose column (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Depletion of T cells and neutralization of cytokines in vivo

Mice were injected i.p. with specified combinations of mAb. Anti-CD4 and anti-CD8 were administered at 100 μg/dose, while anti-TNFα, anti-IFNγ, anti-GM-CSF, and anti-IL-10 were administered at 1 mg/dose. Mice were given one dose of the antibody weekly until the end of the experiment. Depletion of greater than 95% of CD4 and CD8 population in the spleen was confirmed by flow cytometry. Neutralization of cytokines was confirmed by infecting naïve mice with a sublethal dose of Hc plus administering one dose of mAb at the time of infection. Mortality observed 7-10 days post infection indicated sufficient neutralization of the cytokine.

Isolation of leukocytes from spleens and lungs

Lungs and spleens were teased apart with the frosted ends of two glass slides and washed three times in HBSS. Splenocytes were diluted in 0.04% trypan blue saline solution and leukocytes were enumerated. To obtain lung leukocytes, a stock solution was prepared using a 1:10 ratio of 10X phosphate-buffered saline (PBS) to Percoll™ (Amersham Biosciences). The stock solution was further diluted with HBSS to 40% and 70%. Separation gradients were prepared by resuspending the lung homogenates in 4 ml of the

40% solution. The cells were carefully layered on top of 2 ml of 70% solution in a round bottom tube. The gradients were centrifuged for 20 minutes at 800 xg. The leukocytes were collected from the interphase between the two layers, washed in HBSS, and enumerated as described for splenocytes.

Flow Cytometry

Antibodies conjugated to allophycocyanin (APC), fluoroscein isothiocyanate (FITC), or phycoerthyrin (PE) were used for all flow cytometry experiments. CD3-APC, CD4-FITC, CD8-FITC, CD45R/B220-FITC, CD11b-APC (Mac1), Ly-6G-APC (Gr1), NK1.1-FITC, IL-10-PE and isotype controls IgG2b-FITC, IgG2a-APC, and Hamster Ig, group 1, κ isotype were purchased from BD Pharmingen, San Diego, CA. To determine the phenotype of leukocytes obtained from lungs and spleens of infected mice, 10^6 cells were washed in 1% bovine serum albumin (BSA) in PBS. The cells were incubated with 0.2 μg of antibody for 10 minutes at 4°C. The cells were washed and fixed in 1% paraformaldehyde in PBS until analysis. To evaluate intracellular cytokine production, 10^6 cells were stained for surface markers as described above. Cells were washed and resuspended in Cytofix/Cytoperm™ (BD Pharmingen) for 20 minutes on ice. Cells were washed in Perm/Wash™ buffer (BD Pharmingen) and incubated with 0.2 μg of anti-IL-10-PE in Perm/Wash™ buffer at room temperature. The cells were washed and resuspended in Perm/Wash™ buffer until analysis. Fluorescence was measured using a FACScaliber flow cytometer (BD Pharmingen).

Statistical Analysis

To analyze differences between groups, a one-way analysis of varance (ANOVA) was performed. If the data achieved normality, the Tukey test was used to allow multiple comparisons between different groups. Otherwise, the Wilcoxon rank-sum test was used.

Literature Cited

1. Zahrt, T. C. 2003. Molecular mechanisms regulating persistent Mycobacterium tuberculosis infection. *Microbes Infect 5:159.*

2. Retallack, D. M., and J. P. Woods. 1999. Molecular epidemiology, pathogenesis, and genetics of the dimorphic fungus Histoplasma capsulatum. *Microbes Infect 1:817.*

3. Schwarz, J. 1981. *Histoplasmosis*. Praeger Publishers, New York.

4. Deepe, G. S., Jr. 1994. The immune response to Histoplasma capsulatum: unearthing its secrets. *J Lab Clin Med 123:201.*

5. Woods, J. P. 2002. Histoplasma capsulatum molecular genetics, pathogenesis, and responsiveness to its environment. *Fungal Genet Biol 35:81.*

6. Deepe, G. S., Jr., and W. E. Bullock. 1992. Histoplasmosis: A Granulomatous Inflammatory Response. In *Inflammation: Basic Principles and Clinical Correlates*, Chapter 47. J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds. Raven Press, Ltd, New York, p. 733.

7. Maresca, B., and G. S. Kobayashi. 2000. Dimorphism in Histoplasma capsulatum and Blastomyces dermatitidis. *Contrib Microbiol 5:201.*

8. Newman, S. L., L. Gootee, R. Morris, and W. E. Bullock. 1992. Digestion of Histoplasma capsulatum yeasts by human macrophages. *J Immunol 149:574.*

9. Newman, S. L. 1999. Macrophages in host defense against Histoplasma capsulatum. *Trends Microbiol 7:67.*

10. Strasser, J. E., S. L. Newman, G. M. Ciraolo, R. E. Morris, M. L. Howell, and G. E. Dean. 1999. Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by Histoplasma capsulatum. *J Immunol 162:6148.*

11. Deepe, G. S. 1992. Histoplasma, Infection and Immunity. In *Encyclopedia of Immunity*. I. M. Roitt, and P. J. Delves, eds. WB Saunders, London, p. 681.

12. McKinsey, D. S. 1998. Histoplasmosis in AIDS: Advances in Management. *AIDS Patient Care STDS 12:775.*

13. Edwards, L. B., and e. al. 1969. An atlas of sensitivity to tuberculin, PPB-B and histoplasmin in the United States. *Am Rev Respir Dis 99:Suppl:1.32.*

14. Dietrich, P. Y., P. Pugin, C. Regamey, and J. Bille. 1986. Disseminated histoplasmosis and AIDS in Switzerland. *Lancet 2:752.*

15. Mandell, W., D. M. Goldberg, and H. C. Neu. 1986. Histoplasmosis in patients with the acquired immune deficiency syndrome. *Am J Med 81:974.*

16. Keath, E. J., G. S. Kobayashi, and G. Medoff. 1992. Typing of Histoplasma capsulatum by restriction fragment length polymorphisms in a nuclear gene. *J Clin Microbiol 30:2104.*

17. Cain, J. A., and G. S. Deepe, Jr. 1998. Evolution of the primary immune response to Histoplasma capsulatum in murine lung. *Infect Immun 66:1473.*

18. Allendorfer, R., G. D. Brunner, and G. S. Deepe, Jr. 1999. Complex requirements for nascent and memory immunity in pulmonary histoplasmosis. *J Immunol 162:7389.*

19. Gomez, A. M., W. E. Bullock, C. L. Taylor, and G. S. Deepe, Jr. 1988. Role of L3T4+ T cells in host defense against Histoplasma capsulatum. *Infect Immun 56:1685.*

20. Deepe, G. S., Jr. 1994. Role of CD8+ T cells in host resistance to systemic infection with Histoplasma capsulatum in mice. *J Immunol 152:3491.*

21. Deepe, G. S., Jr. 1988. Protective immunity in murine histoplasmosis: functional comparison of adoptively transferred T-cell clones and splenic T cells. *Infect Immun 56:2350.*

22. Lane, T. E., G. C. Otero, B. A. Wu-Hsieh, and D. H. Howard. 1994. Expression of inducible nitric oxide synthase by stimulated macrophages correlates with their antihistoplasma activity. *Infect Immun 62:1478.*

23. Lane, T. E., B. A. Wu-Hsieh, and D. H. Howard. 1993. Gamma interferon cooperates with lipopolysaccharide to activate mouse splenic macrophages to an antihistoplasma state. *Infect Immun 61:1468.*

24. Zhou, P., M. C. Sieve, J. Bennett, K. J. Kwon-Chung, R. P. Tewari, R. T. Gazzinelli, A. Sher, and R. A. Seder. 1995. IL-12 prevents mortality in mice infected with Histoplasma capsulatum through induction of IFN-gamma. *J Immunol 155:785.*

25. Allendoerfer, R., and G. S. Deepe, Jr. 1997. Intrapulmonary response to Histoplasma capsulatum in gamma interferon knockout mice. *Infect Immun 65:2564.*

26. Zhou, P., G. Miller, and R. A. Seder. 1998. Factors involved in regulating primary and secondary immunity to infection with Histoplasma capsulatum: TNF-alpha plays a critical role in maintaining secondary immunity in the absence of IFN-gamma. *J Immunol 160:1359.*

27. Allendoerfer, R., and G. S. Deepe, Jr. 1998. Blockade of endogenous TNF-alpha exacerbates primary and secondary pulmonary histoplasmosis by differential mechanisms. *J Immunol 160:6072.*

28. Deepe, G. S., Jr., R. Gibbons, and E. Woodward. 1999. Neutralization of endogenous granulocyte-macrophage colony-stimulating factor subverts the protective immune response to Histoplasma capsulatum. *J Immunol 163:4985.*

29. Deepe, G. S., Jr., and R. Gibbons. 2000. Recombinant murine granulocyte-macrophage colony-stimulating factor modulates the course of pulmonary histoplasmosis in immunocompetent and immunodeficient mice. *Antimicrob Agents Chemother 44:3328.*

30. Perez, R. L., C. A. Rivera-Marrero, and J. Roman. 2003. Pulmonary granulomatous inflammation: From sarcoidosis to tuberculosis. *Semin Respir Infect 18:23.*

31. Durkin, M., S. Kohler, C. Schnizlein-Bick, A. LeMonte, P. Connolly, J. Goldberg, T. Garringer, and L. J. Wheat. 2001. Chronic infection and reactivation in a pulmonary challenge model of histoplasmosis. *J Infect Dis 183:1822.*

32. Allendoerfer, R., G. D. Brunner, and G. S. Deepe, Jr. 1999. Complex requirements for nascent and memory immunity in pulmonary histoplasmosis. *J Immunol 162:7380*